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Der Präsident des Europäischen Patentamts
Im Auftrag

For the President of the European Patent Of

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If no title is shown please refer to the description.
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Method of producing double low restorer lines of Brassica napus having a good agronomic value

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The invention relates to a method of producing a double low restorer lines of Brassica napus for Ogura cytoplasmic male sterility (cms) presenting a radish introgression carrying the Rfo restorer genes deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from Brassica oleracea, and having a good agronomic value characterized by female fertility, a good transmission rate of Rfo and a high vegetative vigour. The invention relates also to a method of forming Brassica napus hybrid seed and progeny thereof and to the use of markers for selection.

Breeding restorer lines for the Ogu-INRA Cytoplasmic Male Sterility (cms) system in rapeseed (Brassica napus L.) has been a major objective during the past few years. Extensive backcross and pedigree breeding were necessary to improve their female fertility and to get double low restorer lines. The so-called « double low » varieties are those low in erucic acid in the oil and low in glucosinolates in the solid meal remaining after oil extraction. However some difficulties can still be encountered in breeding these lines (introgression rearrangements, possible linkage with negative traits) due to the large size of the radish introgression.

The inventors thus assigned themselves the objective of providing a new improved double low restorer line with a good agronomic value.

This objective is obtained by a new method of producing a recombined double low restorer line for the Ogu-INRA cms in rapeseed.

A first object of the present invention relates to a method of producing double low restorer lines of Brassica napus for Ogura cytoplasmic male sterility (cms) presenting radis introgression carrying the Rfo restorer gene deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from Brassica oleracea, and having a good agronomic value characterised by female fertility, a good transmission rate of Rfo and a high vegetative vigour, said method including the step of:

a) crossing double low cms lines of spring Brassica napus comprising a deleted radish insertion with the double low line of spring Drakkar for forming heterozygous restored plants of Brassica napus,

b) irradiating before meiosis the heterozygous restored plants obtained in step a) with gamma ray irradiation,

c) crossing pollen from flowers obtained in step b) with the cms double low spring Wesroona line,

d) testing the progeny for vigour, female fertility and transmission rate of the cms gene,

e) selecting progeny lines.

A method according to claim 1, wherein the irradiation dose in step b) is 65 Gray during 6 mn.

According to one advantageous form of embodiment of the method according to the present invention, the double low cms line of spring Brassica napus of step a) is R211.

R211 is an INRA spring restorer line.

Drakkar is a French spring registered variety.

Wesroona is an Australian spring registered variety.

According to one advantageous form of embodiment of the method according to the present invention, the testing is performed with the combination of five markers selected from PGIol, PGIUNT, PGIint, BolJon and CP418.

Another object of the present invention relates to double low restorer lines of Brassica napus for Ogura cms presenting a Rfo insertion deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from Brassica oleracea, and having a good agronomic value characterised by female fertility, a good transmission rate of Rfo and a high vegetative vigour.

According to one advantageous form of embodiment, the double low restorer lines present a unique combination of five markers selected from PGIol, PGIUNT, PGIint, BolJon and CP418.

Another object of the present invention relates to a method of forming Brassica napus hybrid plants and progeny thereof obtained though the steps of:

a) providing a restorer line produced according to claim 1 and bred to be homozygous,

b) using said restorer line in a hybrid production field as the pollinator,

c) using cms sterile plants in a hybrid production field as the hybrid seed producing plant, and

d) harvesting the hybrid seed from the male sterile plant.

Another object of the present invention relates to seeds of Brassica plant obtained from the methods according to the present invention.

Still another object of the invention relates to seeds of Brassica napus deposited in NCIMB Limited, 23 St Machar Drive, Aberdeen, Scotland, AB24 3RY, UK, on July 4 , 2003, under the reference number NCIMB41183.

Another object of the present invention relates to the use of the combined markers PGIol, PGIUNT, PGIint and BolJon for selecting recombined restorer lines of Brassica napus for Ogura cms presenting a Rfo insertion deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from Brassica oleracea, and having a good agronomic value characterised by female fertility, a good transmission rate of Rfo) and a high vegetative vigour.

Such markers are represented in the following figures for the R2000 line.

According to one advantageous form of embodiment, the present invention relates to:

- The marker PGIol which is amplified using the primers: PGIol U and PGIol L
  (PGIol U: 5'TCATTTGATTGTTGCGCCTG3';
  PGIol L: 5'TGTACATCAGACCCGGTAGAAAA3')
- The marker PGIint which is amplified using the primers: PGIint U and PGIint L
  (PGIint U: 5'CAGCACTAATCTTGCGGTATG3';
  PGIint L: 5'CAATAACCCTAAAAGCACCTG3')
- The marker PGIUNT which is amplified using the primers: PGIol U and PGIint L:
  (PGIol U: 5'TCATTTGATTGTTGCGCCTG3';
  PGIint L: 5'CAATAACCCTAAAAGCACCTG3')
- The marker BolJon which is amplified using the primers: BolJon U and BolJon L:
  (BolJon U: 5'GATCCGATTCTTCTCCTGTTG3';
  BolJon L: 5'GCCTACTCCTCAAATCACTCT3')
- The marker CP418 which is amplified using the primers: SG129 U and pCP418 L:
  (SG129 U: cf Giancola et al, 2003 *Theor Appl. Genet. (in press)*
  pCP418 L : 5'AATTTCTCCATCACAAGGACC3')

Another object of the present invention relates to the combination of five markers PGIol, PGIUNT, PGIint, BolJon and CP418 whose sequences are described in the appending figures.

In the annexed drawing that follows, the following abbreviations are used :

Dra	Drakkar
Rel-15-1, E38,R15	R2000
Hete, Hel	heterozygous R211*Drakkar
Darm	Darmor
Bol:	Brassica oleracea
Bra, B.rap:	Brassica rapa
GCPA18-A19, Wes, Aust:	Wesroona
Sam, SamIPGIolSunt5	Samourai
RRH1, ba2c	RRH1

- Figure 1 illustrates Gamma ray Iradiation and F2 production.
- Figure 2 illustrates seed set on 'R211' and 'R2000'.
- Figure 3 illustrates the number of seeds per pod of different lines.
- Figure 4 illustrates PGIol primer localisation on the segment of PGI sequence from Data Base. In that figure:

PGIol: - primer PGIol U (named in SGAP: BnPGIch 1 U)
- primer PGIol L (named in SGAP: Bn PGIch 1 L)

PGIint: - primer PGIint U
- primer PGIint L (is out side the sequence, after 500bp).

- Figure 5 illustrates electrophoresis gel of PGI-2 gene (PGIol), PCR marker and SG91, a PCR marker close to Rfo.
- Figure 6 illustrates Pgi-2 segment of DNA amplified by PCR with PGIol primers.
- Figure 7 illustrates digestion of the PCR product PGIol by Mse1.

In that figure:

Sam and Darm has a 75bp band.

Drak, R211.Dk and R2000 showed a 70pb one (Acrylamide 15%).

8 was similar to Samourai (75bp) ; mix with Drakkar (70pb) it allowed the visualisation of the two bands.

- Figure 8 illustrates electrophoresis gel of PGIUNT marker.

In that figure:

PGIUNT band (about 950bp) is present in B.oleracea, B.rapa cv Asko, maintainer and restored lines except in 'R211'.

There is no amplification in radish and Arabidopsis.

In various Brassica genotypes only one band was amplified. Size band are similar but sequences are different.

Sequence of Restored lines RRH1 and R113 are homologous to B.rapa sequence.

Sequence of R2000 is homologous to Drakkar and B.oleracea sequences.

- Figure 9 illustrates electrophoresis gel of PGIint PCR marker.

In that figure PGIint of radish line 7 is of about 950bp. This band is the same as in the restored RRH1 and R113. It is not found in R211. It is not either in R2000. However the PGIint band is of a similar size in the various Brassica species.

- Figure 10 illustrates PGIint and PGIUNT of B.oleracea group sequences.

- Figure 11 illustrates PGIint and PGIUNT of B.rapa group sequences: the numbers indicates the base substitutions between the two sequences and del is for a deletion in B. oleracea sequence.

- Figure 12 illustrates comparison between B.olearecea and B.rapa PGI sequences.

- Figure 13 illustrates the comparison of Drakkar, Wesroona R2000, B.oleracea and R.rapa PGI sequence comparison.

- Figure 14 illustrates comparison of RRH1, R113, which is similar to B.rapa and different from B.oleracea.

- Figure 15 illustrates comparison of RRH1, R113 and Sam B.rapa on one side and R2000, Drakkar and Wesroona and B.oleracea on the other side.

- Figure 16 illustrates Bo1Jon PCR marker.

- Figure 17 illustrates CP418 marker.

In that figure, the CP418 band (about 650bp) is specific to the B.oleracea genome. It is present in B.ol, B.napus (Samourai, Drakkar, Pactol and the herterozygous R2111*Dk). It is absent from the restored rapeseed (RRH, R113 and R211). It is present in the homozygous R2000.

- Figure 18 illustrates summary markers table.

It should be understood, however, that the example is given solely by way of illustration of the object of the invention, of which they in no way constitute a limitation.

Example I: method of producing a double low restorer line of Brassica napus for Ogura cytoplasmic male sterility (cms) presenting a radish introgression, carrying the Rfo restorer gene deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from Brassica oleracea, and having a good agronomic value characterised by female fertility, a good transmission rate of Rfo) and a high vegetative vigour.

Materials and methods :

Genotypes: The 'R211' line with a deleted radish insertion was crossed to the spring low GLS rapeseed 'Drakkar' to produce a F1 progeny ('R211*Dk'). The spring low GLS cms line 'Wesroona' (australian origin) was used for following crosses. Were used as control in molecular analyses: Winter restored lines derived from 'Samourai' carrying the complete ('RRH1') or incomplete ('R113',) introgression as well as European radish line7, Asiatic restored radish D81, wild radish, Brassica oleracea, and B.rapa cv Asko, Arabidopsis thaliana.

Gamma ray irradiation: Whole flowering plants were treated with gamma rays from a Co60 source in a controlled area. Subletal dose fo 65 Gray was applied before meioses. The chosen dose was used for further two experiments on 35 plants.

Testcrosses and F2 production: Irradiated plants were transferred in an insectproof greenhouse after removing flower buds larger than 2 mm. The irradiated F1 progeny was used to handpollinate the cms 'Wesroona' line. The restored derived F1' plants were allowed to produce F2 families harvested individually and precisely sown in a field assay along with non irradiated controls (Fig 1).

Phenotypic selection: Three visual criteria were scored (on a 1 to 5 scale) over 2 years in field assays, on 1200 F2 offsprings plus 44 controls (82 330 quoted plants):

1-Vegetative vigour,

2- Normality of the ratio of fertile /sterile plants in the F2 segregation, and

3- Female fertility (pod development and seed set).

Advanced selfed generations of the selected families were obtained either in field or greenhouse and produced homozygous lines (F4) for further analysis.

Isozyme analysis was performed as in (Delourme R. and Eber F. 1992. *Theor Appl Genet* 85: 222-228), marker development from (Fourmann M et al 2002. *Theor Appl. Genet.* 105:1196-1206.): PCR products are validated by sequencing.

Alignments were made using Blast Ncbi and Uk Crop Net Brassica DB and the Multialin software INRA Toulouse.

Method :

We choose one low GLS spring homozygous restorer line, 'R211', already exhibiting deletions in the introgression (Delourme R. and Eber F. 1992. *Theor Appl Genet* 85: 222-228. Delourme R et al 1998. *Theor Appl Genet* 97: 129-134. Delourme R. et al 1999. *10th Int. Rapeseed Congress, Canberra.).* Several molecular markers are missing on either side of Rfo, such as spATCHIA (Fourmann M et al 2002. *Theor Appl. Genet.* 105:1196-1206), spSG91 (Giancola S et al 2003 *Theor Appl. Genet. (in press)*). 'R211' lost the isozyme expression of the Pgi-2 allele of the radish gene but also the one of Pgi-2 allele of B.oleracea genome (1,2). Moreover, the homozygous 'R211' shows linked negative traits such as low vigour and very poor seed set. We hypothesised that these plant lack a rapeseed chromosomal segment. The fertile ratio in F2 progenies derived from this material is lower than expected (64% instead of 75%). We initiated the program from this 'R211' line and tried to force recombination between the Rfo carrying introgression from this deleted line and the rapeseed homologous chromosome from a double low B. napus line.

Ionising irradiation is known to induce chromosomal rearrangements by double strand breaks followed by aberrant rejoining of the ends.Gamma-ray irradiation was used on a heterozygous F1 derived from the 'R211' line to induce chromosome breaks, just before meiosis, aiming at a recombination of the deleted radish introgression in the rapeseed genome.

Results :

Very few families were at the best score for the three criteria out of 1200 F2 families tested.

Only one, 'R2000', proved to produce a normal ratio of fertile plants per selfed progeny with a stable recovery of good agronomic traits such as a good female fertility, with a normal seed set compared to 'R211' (Fig 2 and 3). This family was obtained from a 6 mn irradiation treatment.

Glucosinolate analysis confirmed its low content.

In figure 2 (Seed set on 'R211' and 'R2000') R2000 showed normal inflorescences, with a normal looking architecture.

In figure 3 (Number of seeds per pod), we observe:

- on the best 'R2000' F4 families in self pollination (Selfings) and in testcrosses
- on 'Pactol' cms line on rapeseed and 'R211' controls.

Example II: selection of markers in the Pgi-2 gene

PGI isoenzyme analysis: 'R2000' progeny expressed the rapeseed Pgi-2 allele from B. oleracea genome, originally lost in 'R211'.

Three PCR markers were defined to characterise the R2000 family compared to the known restorer rapeseed RRH1 and R113.

1) PGIol marker was developed from the BrassicaDB sequences to be specific to the Brassica genome. There is no amplification in radish nor in Arabidopsis th. , but only in Brassica, with one 278 bp band.

2) PGIint marker amplified a longer part of the Pgi-2 gene, allowing clear distinction between the various tested species Brassica, Raphanus and Arabidopsis. The species B.rapa and B.oleracea were not distinguished by the band size, but by their PGINT band sequence.

3) PGIUnt marker, a combination of the PGI ol U and PGI int L primers. This marker had the specificity of the PGIol marker but amplifying a longer part as for PGIint one.

II.1 PGIol marker

With the PGIol primers, the 'R211' parental line showed no amplification, while the spring tested lines showed a 278bp band. Its DNA sequence is homologous to the PGI-2 sequences from the Crop Net UK DB in Brassica species and from previous work in our group (named SGAP sequences) (Localisation of the primers SG PGI chou, Fig 4).

It was ortholog of the clone MJB21-12, on the chromosome V, (34543bp) in Arabidopsis (NCBI DB).

PGIol plus SG91 to set an Homozygocity test:

The combined use of two sets of primers in a mix PCR, PGIol marking the Pgi-2 gene and SG91 (from S. Giancola et al, Giancola S et al 2003 *Theor Appl. Genet.*

(in press)), a very close marker to the Rfo gene, was set up to discriminate homozygous from heterozygous plant among the fertile plants segregating in F2 progenies derived from 'R211'.

One family R2000 showed no difference between homozygote and heterozygote offsprings:

The Pgi-2 gene is present in the R2000 homozygote, which is not the case for the parental homozygous R211.

In figure 5 (PGIol and SG91 PCR markers):

SG91 is located on the radish introgression very close to Rfo

The homozygous 'R2000' family has recovered the PGIol band.

DNA sequence of the band confirmed the homology with the known Arabidopsis and Brassica Pgi-2 sequence. Control genotypes (Drakkar, Pactol, and, Samourai, Darmor) had the same pattern on the gel. Sequence of this common band allowed to confirm their high homology as they were quasi similar except one base substitution.

The homozygous 'R2000' family has recovered the PGIol band having the characteristic of the Drakkar group of cultivars. It was distinct from those of the Samourai group, including the known restorers.

This amplified part of the Pgi-2 is very conserved and hardly any differences were shown among the various genotypes. A longer part of Pgi-2 gene was investigated.

II.2 PGIUNT and PGIint markers

Electrophoresis Patterns of PCR products :

PGIUNT marker: A second reverse primer, PGIint L, was designed further down the Pgi-2 sequence, to amplify as well conserved and as variable regions of the gene. When used with the PGIol U primer, it amplifies a 950bp band only in Brassica genomes.

R211 didn't show any band, The homozygous 'R2000' showed the PGIUNT band as in the Drakkar parent.

In figure 8 (PGIUNT marker) :

PGIint marker amplified a longer part of the Pgi-2 gene, allowing clear distinction between the tested species. B.rapa and B.oleracea were not distinguished by the band size, but by their PGIint sequence. All tested restored genotypes, but the

'R211' line, exhibited the European radish band and one Brassica band, homologous to the B.rapa one.

The homozygous 'R2000' didn't show the radish PGIint band, as in the deleted 'R211' parental line, but showed one Brassica band, homologous to the B. oleracea one.

Electrophoresis of PGIint marker is represented in figure 9.

Sequence analysis:

Comparison of the PGI sequences from the data bases.

A PGI segment of about 490bp is known.

Sequences of a segment of about 490bp from different genotypes (B. oleracea, B. rapa, B. napus) have been studied in our laboratory group and some sequences were given to Brassica Crop Net DB: EMAF25875 to 25788 by M.Fouramnn (4) These sequences are very conserved.

Comparison of the B. rapa et B.oleracea species PGI sequences:

Comparison between PGI sequences we have obtained from the tested genotypes of B.oleracea and B.rapa species, showed that they were distinct by 19 base substitutions. Theses substitutions allowed to distinguish PGIint sequences from the other tested genotypes of rapeseed, homologous to either B.rapa cv Asko (RRH1 and R113) or B.oleracea (Drakkar, R211*DK but also R2000).

Example III: selection of marker in a region close to Rfo

Markers surrounding the Rfo gene in the radish insertion were determined in order to facilitate the Rfo gene cloning (Desloires S et al 2003 *EMBO reports* 4, 6:588-594). One of these, the SG129 PCR marker was located very close to Rfo (Giancola S et al 2003 *Theor Appl. Genet. (in press)*): it co-amplified distinct bands in B.oleracea and B.rapa genomes of B.napus, but the radish band was very difficult to see on a gel.

The target SG129 sequence was ortholog of a clone (AC011OOO, at the locus F16P17) in Arabidopsis thaliana. This clone overlapped an Arabidopsis adjacent contig clone (AC07190).

From the Brassica Crop Net DB, we found one B.oleracea clone, (EMBH448336, 764bp) blasting with the begining of the A011000, and a second B.oleracea clone

(EMBH53971), distant from about 300bp on the Arabidopsis map, that blasted with the end of ACO7190.

We designed a new PCR marker, BolJon, between the two B.oleracea clones. We verified that it allowed amplification of a specific PCR bands in the different genotypes compared here.

In figure 22 (electrophoresis gel of BolJon PCR products):

- In Arabidopsis, a BolJon 815bp band was amplified, homologue to the overlapping segment of the contigs.
- In Brassiceae diploid species, BolJon marker showed distinct bands: one of 950bp in B.oleracea and one of 870bp in B.rapa. It showed that the two B.oleracea clones (EMBH53971 and EMBH448336) are in sequence continuity in Brassica genome as it is for the ortholog sequences in Arabidopsis.
- In B.napus, these two bands are co-amplified in the maintainer lines, Samourai or Drakkar.
- In radish line7, one BolJon band was amplified of about 630 bp long. The band of the restored radish cmsRd81 was slightly smaller.
- In all the restored rapeseed lines, one of the BolJon bands was of the same size as the radish line7. BolJon is a marker of the radish introgression.
- The homozygous restored rapeseed lines, 'RRH1', 'R113' and also 'R211', only showed the B.rapa band and the 630bp radish band bp suggesting the B.oleracea ortholog of the target gene is absent or has been modified when the radish segment of chromosome was inserted into the rapeseed B.oleracea constitutive genome.

'R2000' homozygote plants showed radish PCR BolJon, plus the two Brassica BolJon bands, again having recovered the B.oleracea one, lost in 'R211' and other restorer lines.

We designed a primer, pCP418L, specific of the B.oleracea genome in the tested species. With the SG129U primer it amplified only one PCR band (650bp) in the B.oleracea species. (Fig 23)

There was no amplification in B.rapa, in radish, nor in Arabidopsis, but there was a clear CP418 band in B. napus maintainer lines. Its sequence was strictly homologous to the EMBH448336 sequence. This marker was in a very conserved

DNA sequence allowing no polymorphism between genotypes except by presence / absence.

In RRH1, R113 and in R211 there was no CP418 band, indicating as previously that the B.oleracea ortholog of the target gene is absent or has been modified following the radish insertion.

'R2000' homozygote plants showed CP418 band, again having recovered the specific B.oleracea one.

In the present invention, a new recombined low GLS restorer line has been selected with a good female fertility. The poor value of line 'R211' allowed selection in the field for a rare recombination event and characterisation the 'R2000' family.

The homozygous 'R2000' presents a unique combination of the PGIol, PGIUNT, PGIint and BolJon markers when compared with the rapeseed restorer analysed yet:

PGIinT marker showed that the homozygous restored rapeseed lines, RRH1 and R113 presented the European radish band plus one Brassica band, homologous to B.rapa genome. 'R2000' shows no radish band, lost as in its parental deleted line R211, but showed one Brassica band homologous to B.oleracea. The ortholog PGIint sequence in its B.rapa genome is not amplified with this marker in R211 and Drakkar genetic background.

PGIol marker and PGIUNT marker sequences in restored lines RRH1 and R113 were homologous to the B.rapa cv Asko one. In 'R2000', PGIUNT sequence is homologous to B.oleracea. The ortholog PGIUnt sequence in its B.rapa genome is not amplified with this marker in R211 and Drakkar genetic background.

BolJon marker showed that the homozygous restored rapeseed lines, including 'R211' presented the European radish band plus only the B.rapa one. 'R2000' shows the two bands of 'R211' plus the recovered B.oleracea BolJon band.

CP418 marker showed that 'R2000' recovered this conserved B.oleracea segment.

Our hypothesis is that a recombination event took place in the pollen mother cell which gave rise to 'R2000' plants. The deleted radish introgression was then integrated to the normal homologous chromosome segment, carrying the B.oleracea type Pgi-2 gene and BolJon target sequence, characterised by these markers, probably from the Drakkar '00' genome present in the irradiated heterozygous 'R211*DK'.

The pattern observed for BolJon suggests that the recombination event resulted in a particular duplicated region, one from radish and one B. oleracea, in the 'R2000' family.

CLAIMS

1. A method of producing double low restorer lines of Brassica napus for Ogura cytoplasmic male sterility (cms) presenting radis introgression carrying the Rfo restorer gene deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from Brassica oleracea, and having a good agronomic value characterised by female fertility, a good transmission rate of Rfo and a high vegetative vigour, said method including the step of:
   a) crossing double low cms lines of spring Brassica napus comprising a deleted radish insertion with the double low line of spring Drakkar for forming heterozygous restored plants of Brassica napus,
   b) irradiating before meiosis the heterozygous restored plants obtained in step a) with gamma ray irradiation,
   c) crossing pollen from flowers obtained in step b) with the cms double low spring Wesroona line,
   d) testing the progeny for vigour, female fertility and transmission rate of the cms gene,
   e) selecting progeny lines.

2. A method according to claim 1, wherein the irradiation dose in step b) is 65 Gray during 6 mn.

3 A method according to claim 1 wherein the double low cms line of spring Brassica napus of step a) is R211.

4. A method according to claim 1 wherein the testing is performed with the combination of five markers selected from PGIol, PGIUNT, PGIint, BolJon and CP418.

5. Double low restorer lines of Brassica napus for Ogura cytoplasmic male sterility (cms) presenting a Rfo insertion deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from Brassica oleracea, and having a

good agronomic value characterised by female fertility, a good transmission rate of Rfo and a high vegetative vigour.

6. Double low restorer lines of Brassica napus according to claim 5, wherein they present a unique combination of five markers selected from PGIol, PGIUNT, PGIint, BolJon and CP418.

7. Brassica napus hybrid plants and progeny thereof obtained through the steps of:

a) providing a restorer line produced according to claim 1 and bred to be homozygous,

b) using said restorer line in a hybrid production field as the pollinator,

c) using cms sterile plants in a hybrid production field as the hybrid seed producing plant, and

d) harvesting the hybrid seed from the male sterile plant.

8. The seeds of Brassica plant developed from the Brassica line obtained in claim 1.

9. The seeds of Brassica napus obtained in claim 7.

10. The seeds of Brassica napus obtained in claims 1 and 2 deposited in NCIMB Limited, 23 St Machar Drive, Aberdeen, Scotland, AB24 3RY, UK, on July 4 , 2003, under the reference number NCIMB41183.

11. Use of the combination of five markers PGIol, PGIUNT, PGIint, BolJon and CP418 for selecting RECOMBINED restorer lines of Brassica napus for Ogura cms presenting a Rfo insertion deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from Brassica oleracea, and having a good agronomic value characterised by female fertility, a good transmission rate of Rfo and a high vegetative vigour.

12. Use according to claim 11, wherein:

- The marker PGIol is amplified using the primers: PGIol U and PGIol L
  (PGIol U: 5'TCATTTGATTGTTGCGCCTG3';
  PGIol L: 5'TGTACATCAGACCCGGTAGAAAA3')
- The marker PGIint is amplified using the primers: PGIint U and PGIint L
  (PGIint U: 5'CAGCACTAATCTTGCGGTATG3';
  PGIint L: 5'CAATAACCCTAAAAGCACCTG3')
- The marker PGIUNT is amplified using the primers: PGIol U and PGIint L:
  (PGIol U: 5'TCATTTGATTGTTGCGCCTG3';
  PGIint L: 5'CAATAACCCTAAAAGCACCTG3')
- The marker BolJon is amplified using the primers: BolJon U and BolJon L:
  (BolJon U: 5'GATCCGATTCTTCTCCTGTTG3';
  BolJon L: 5'GCCTACTCCTCAAATCACTCT3')
- The marker CP418 is amplified using the primers: SG129 U and pCP418 L:
  (SG129 U: cf Giancola et al (5)
  pCP418 L : 5'AATTTCTCCATCACAAGGACC3')

13. Combination of five markers PGIol, PGIUNT, PGIint, BolJon and CP418 whose sequences are described in the appending figures.

Method of producing double low restorer lines of Brassica napus having a good agronomic value

ABSTRACT

A method of producing double low restorer line of Brassica napus for Ogura cytoplasmic male sterility (cms) presenting radish introgression carrying the Rfo restorer gene deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from Brassica oleracea, and having a good agronomic value characterized by female fertility, a good transmission rate of Rfo and a high vegetative vigour. A method of forming Brassica napus hybrid seeds and progeny thereof. The seeds of Brassica napus and use of the combined markers PGIol, PGIunt, PGIint, BolJon and CP418 for selecting.

Fig 1

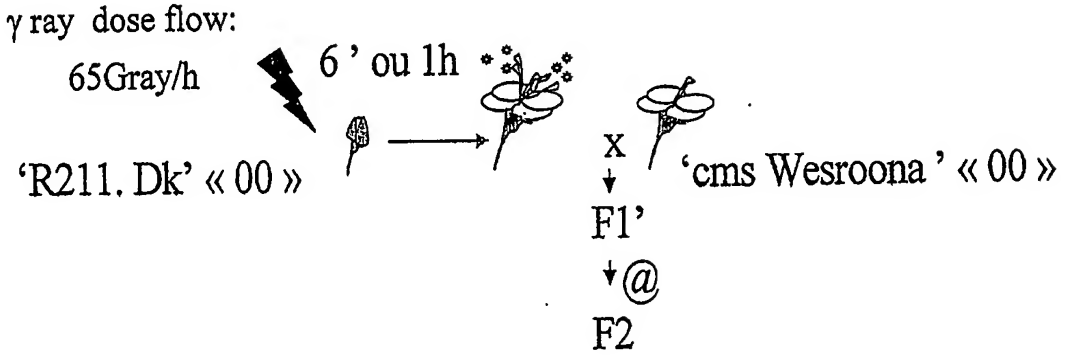

γ ray dose flow:
65Gray/h
6 ' ou 1h
'R211. Dk' « 00 »
x
'cms Wesroona ' « 00 »
F1'
@
F2

Fig 2

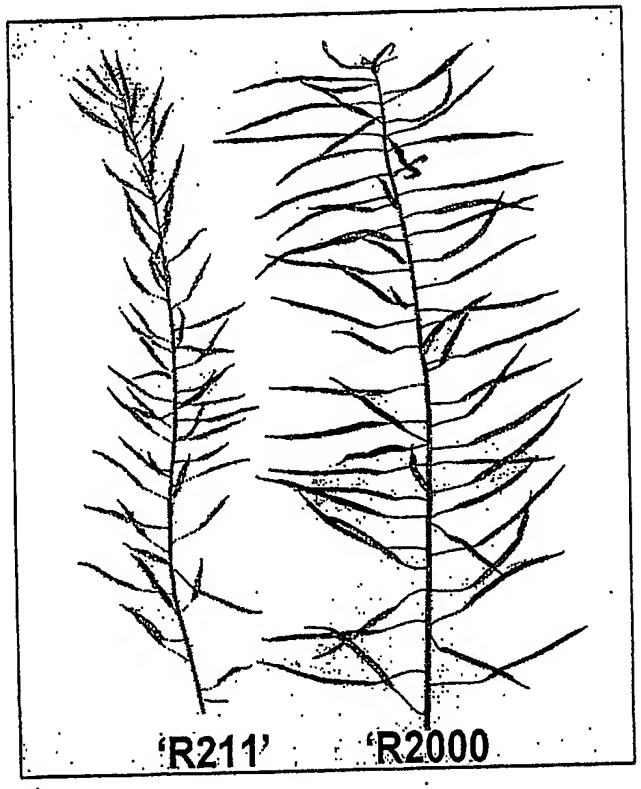


Fig 3

Genotype	Selfings	Test Crosses
Drakkar	29.3	
Pactol	23.1	
R211	11.2	25.5
R2000	**26. 5** (24.0 – 31.1)	**27.0** (24.0 – 28.7)

Fig 4

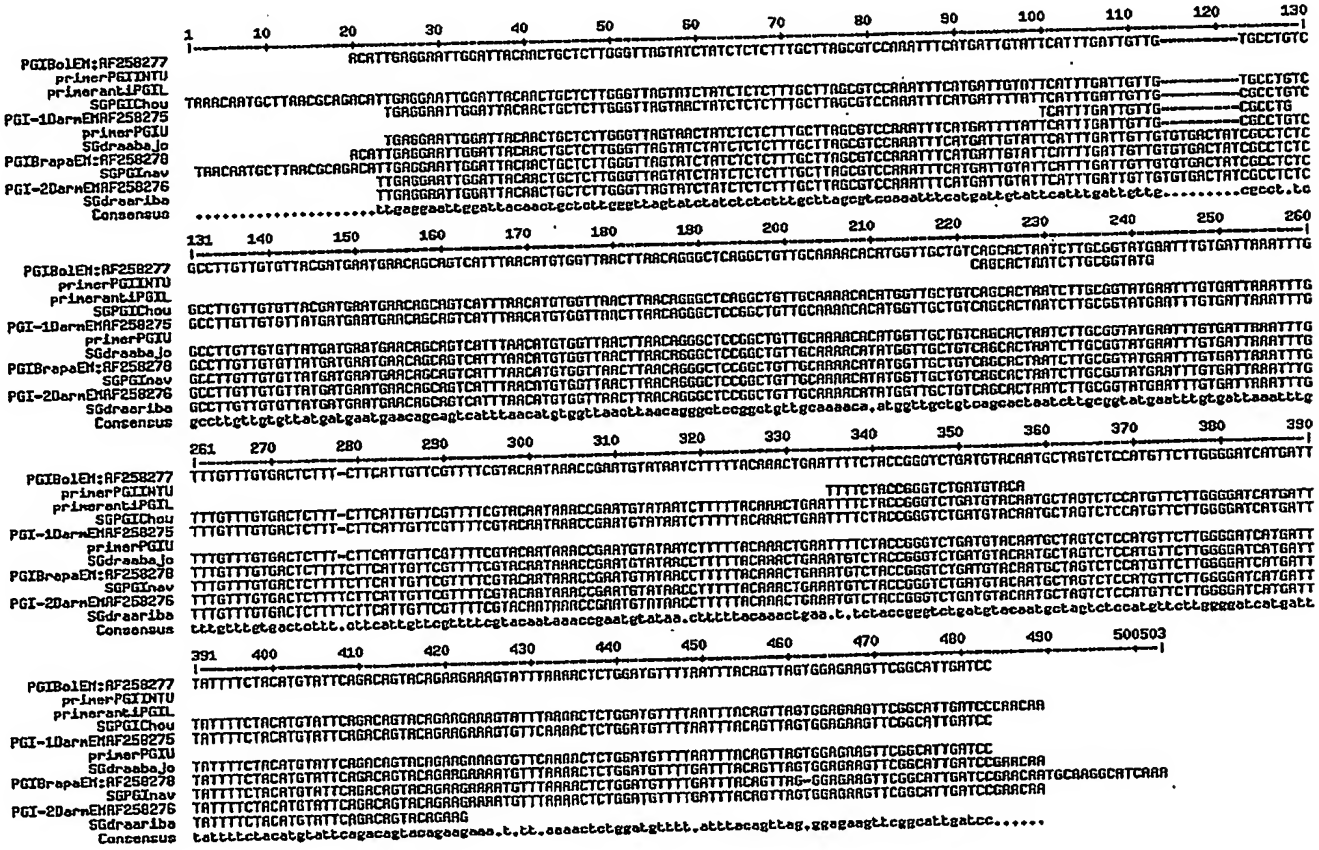

PGIBolEM:AF258277
primerPGIINTU
primerantiPGIL
SGPGIChou
PGI-1DarmEMAF258275
primerPGIU
SGdraabajo
PGIBrapaEM:AF258278
SGPGInav
PGI-2DarmEMAF258276
SGdraariba
Consensus

Fig 5

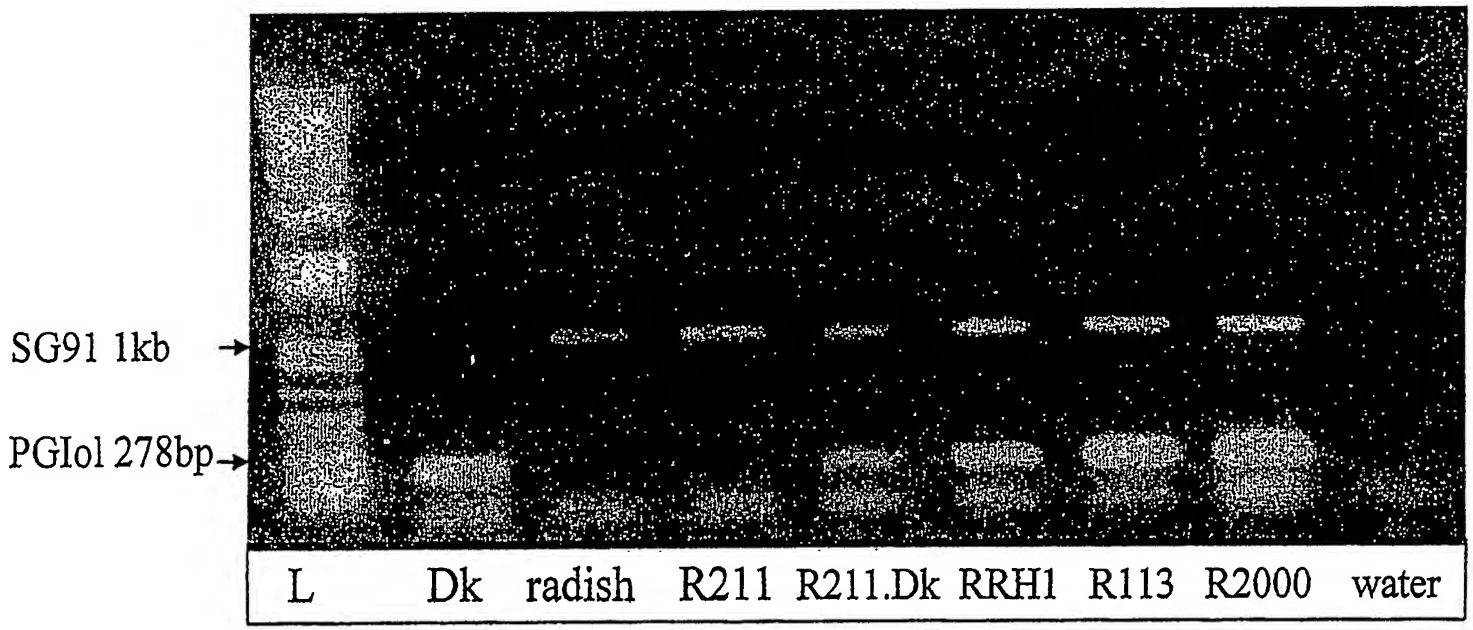
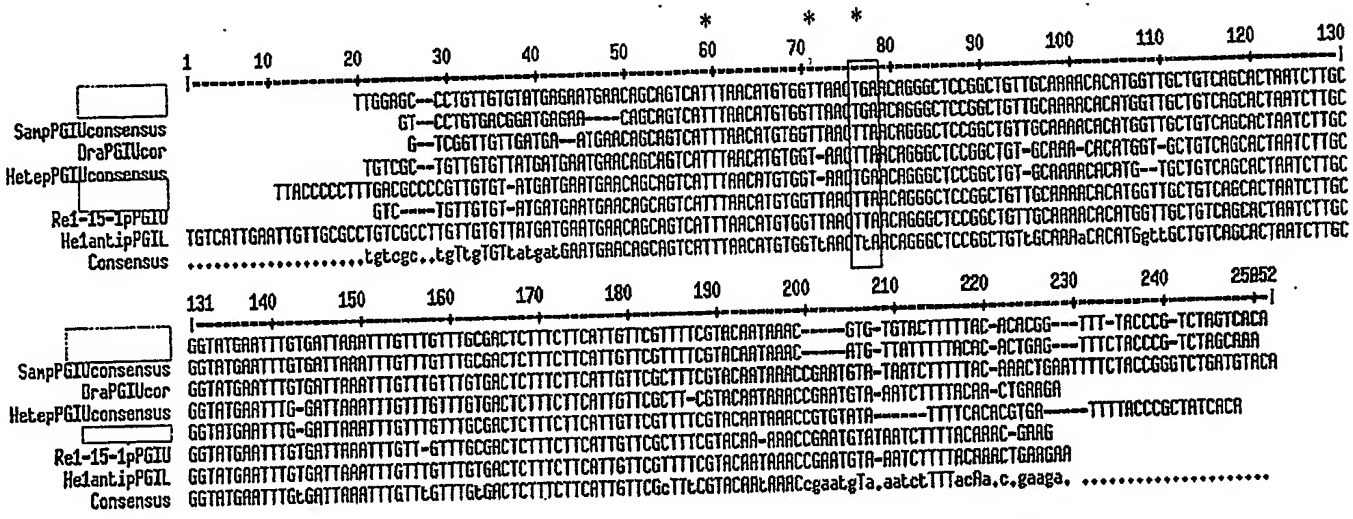
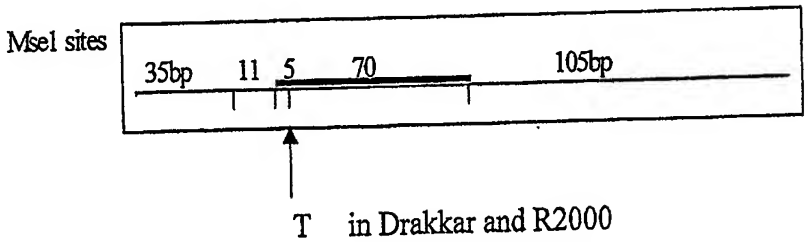

SG91 1kb
PGIol 278bp
L
Dk
radish
R211
R211.Dk
RRH1
R113
R2000
water

Fig 6





T in Drakkar and R2000

MseI restriction enzyme cut DNA sequences at the T/TAA sites (*)

Fig 7

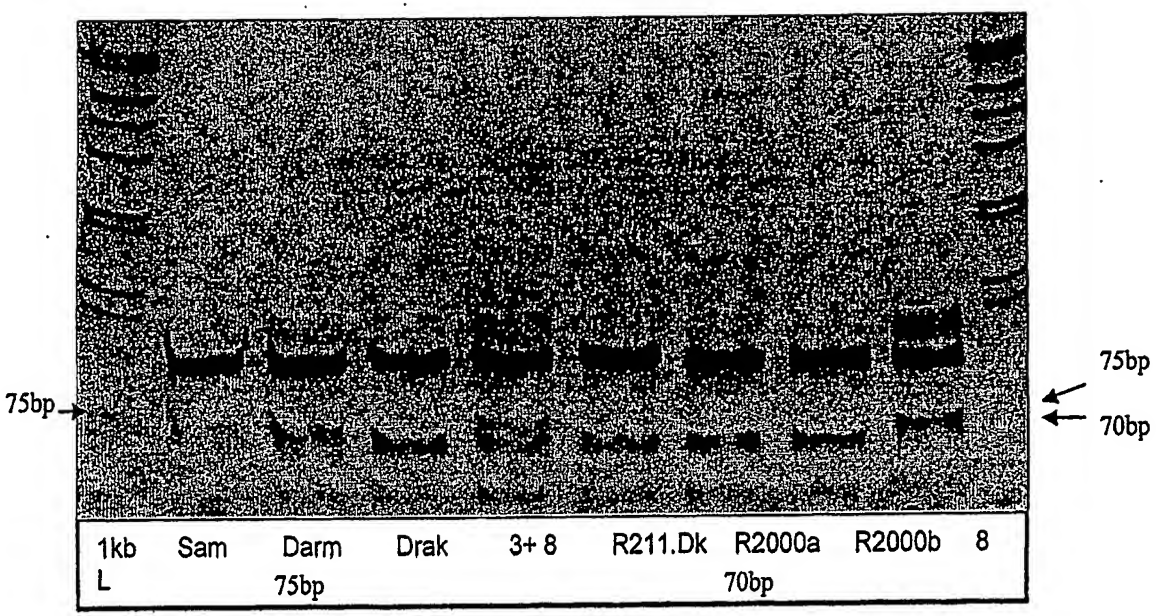

75bp
75bp
70bp
1kb L
Sam
Darm
Drak
3+ 8
R211.Dk
R2000a
R2000b
8
75bp
70bp

Fig 8

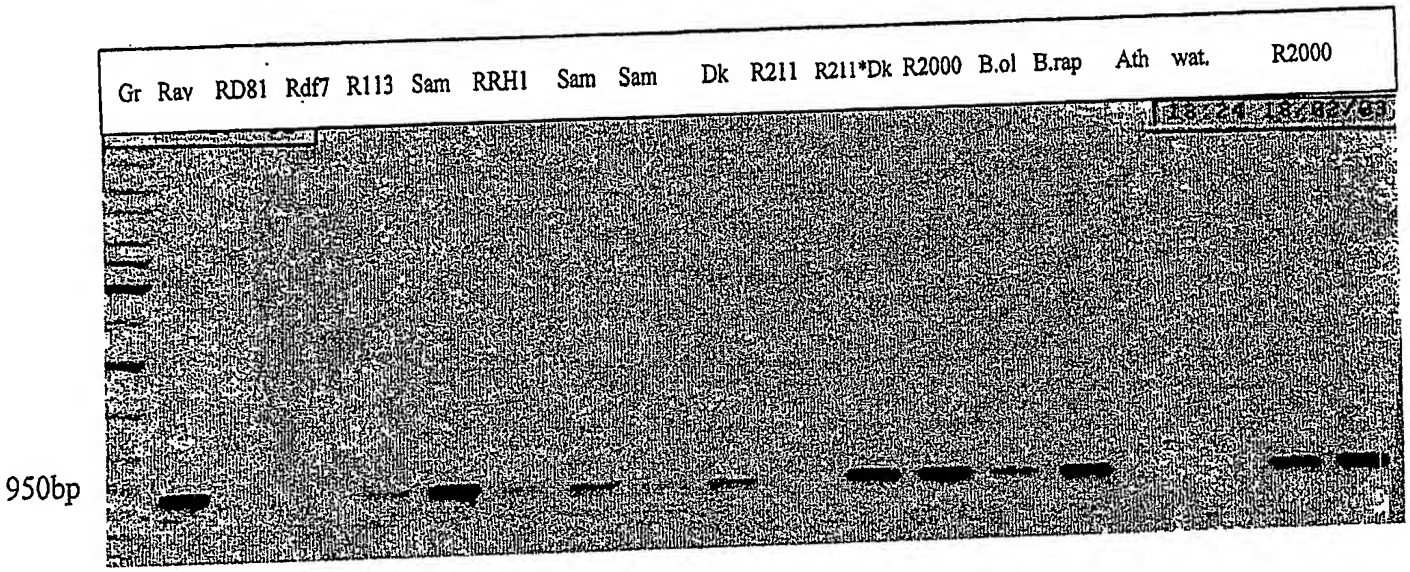

Gr Rav RD81 Rdf7 R113 Sam RRH1 Sam Sam Dk R211 R211*Dk R2000 B.ol B.rap Ath wat. R2000
950bp

Fig 9

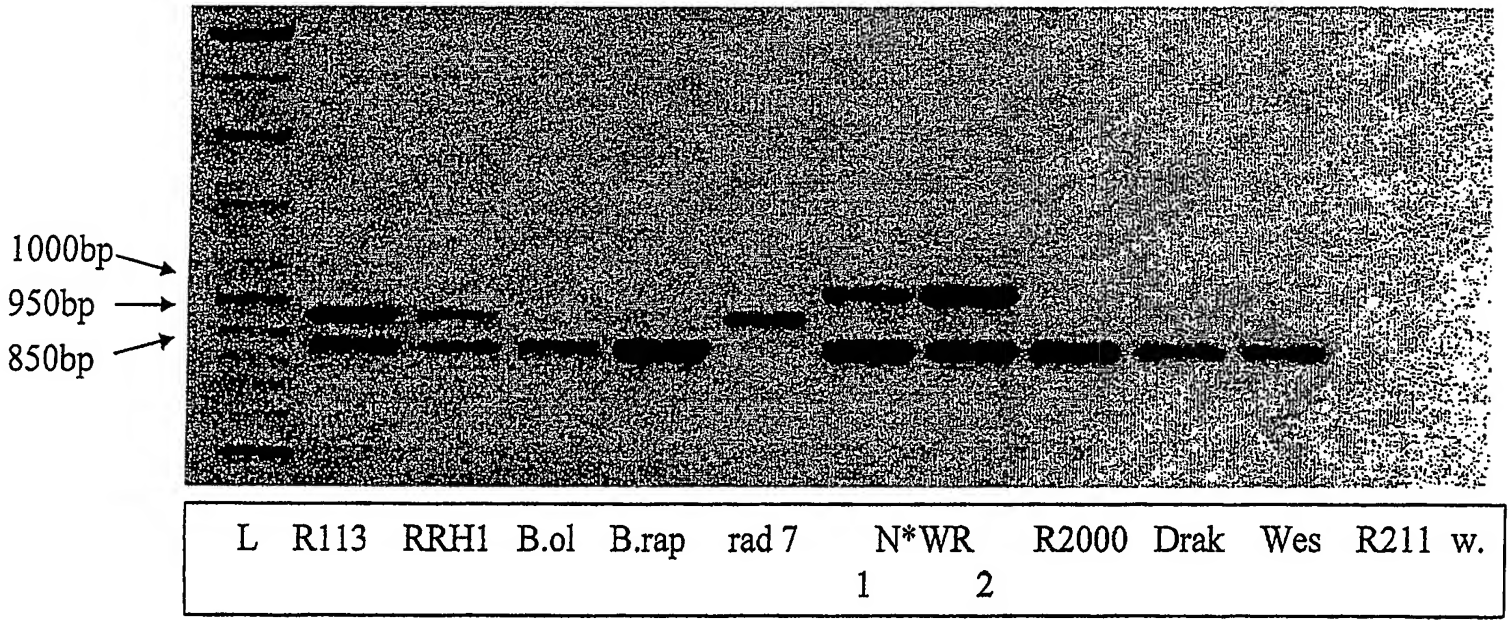

1000bp
950bp
850bp
L
R113
RRH1
B.ol
B.rap
rad 7
N*WR 1
N*WR 2
R2000
Drak
Wes
R211
w.

Fig10

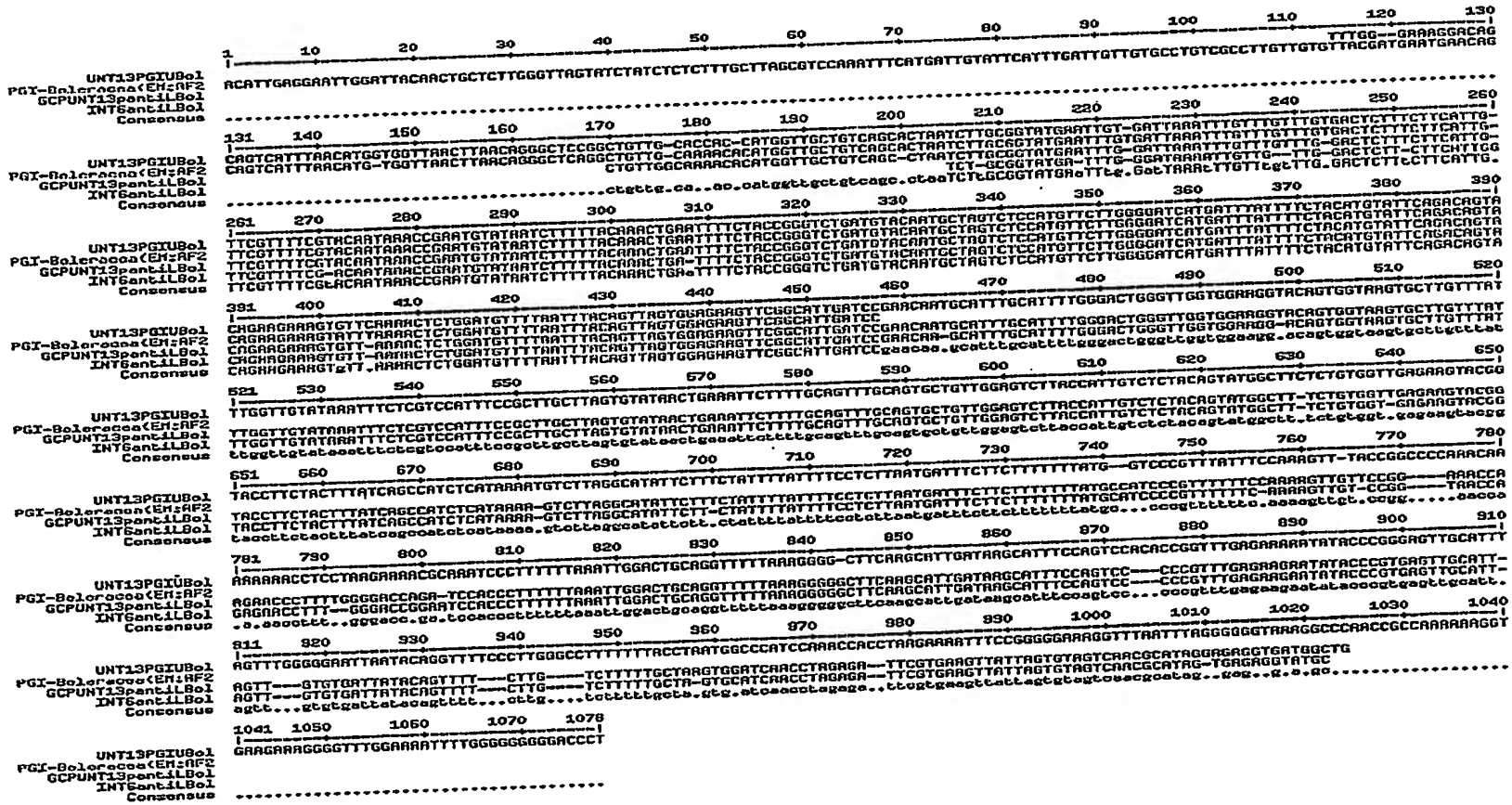

UNT13PGIUBol
PGI-Boleracea(EM:AF2
GCPUNT13pantilBol
INTGantilBol
Consensus

FIG 11

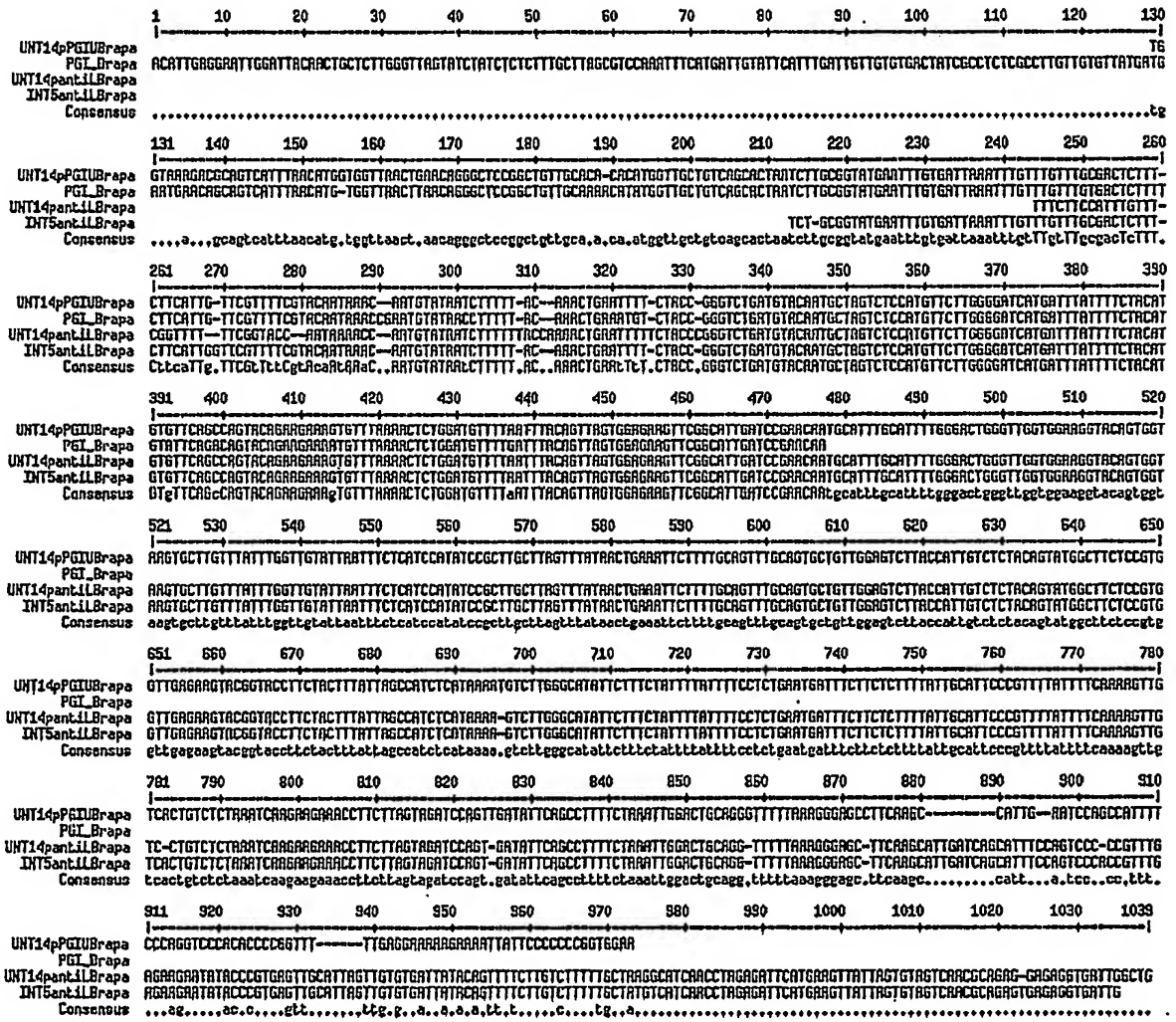
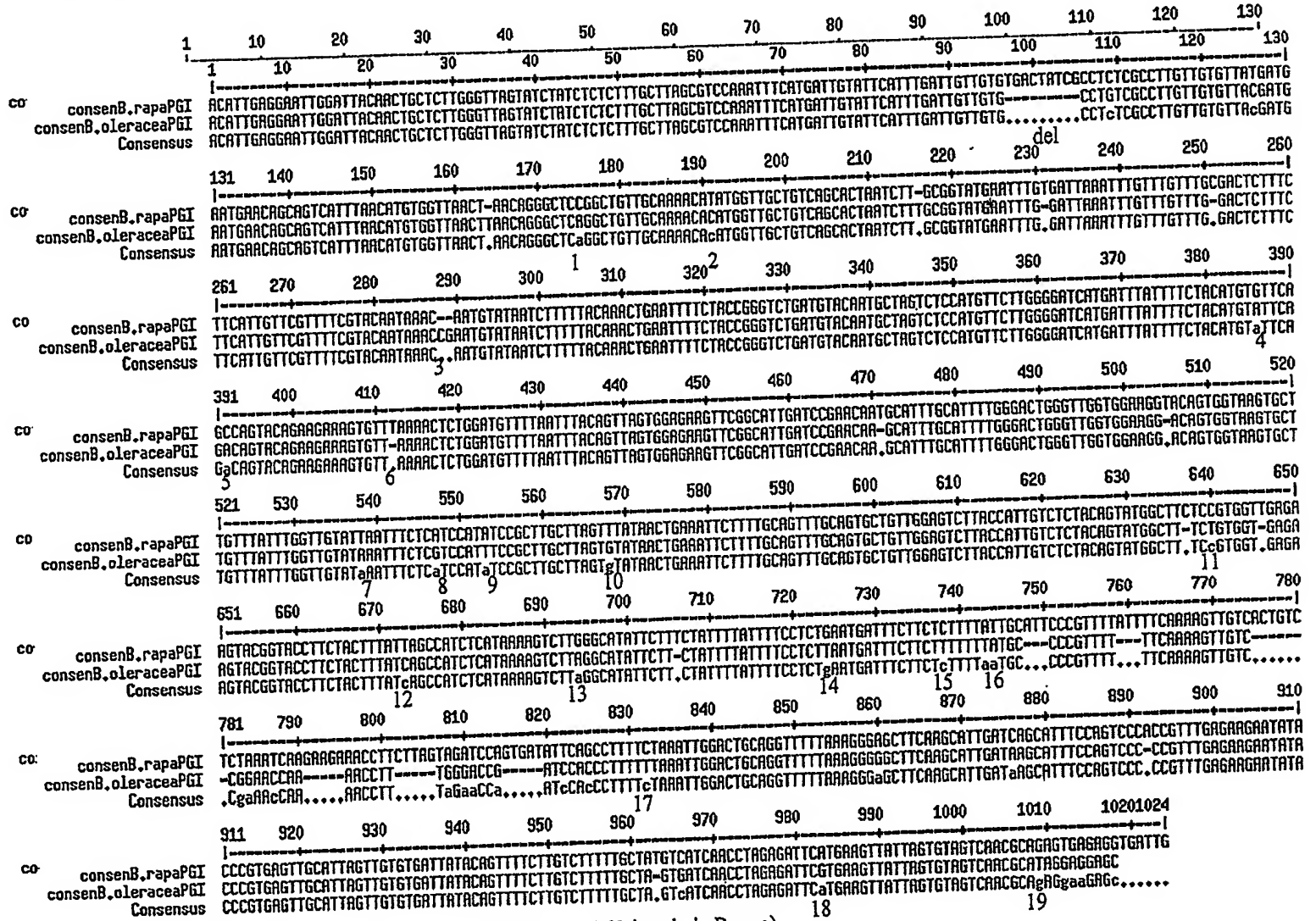

UNT14pPGIUBrapa
PGI_Brapa
UNT14pantilBrapa
INT5antilBrapa
Consensus

FIG 12



* one deletion and 19 substitutions, (n°2 is only in B.rapa)

Fig13

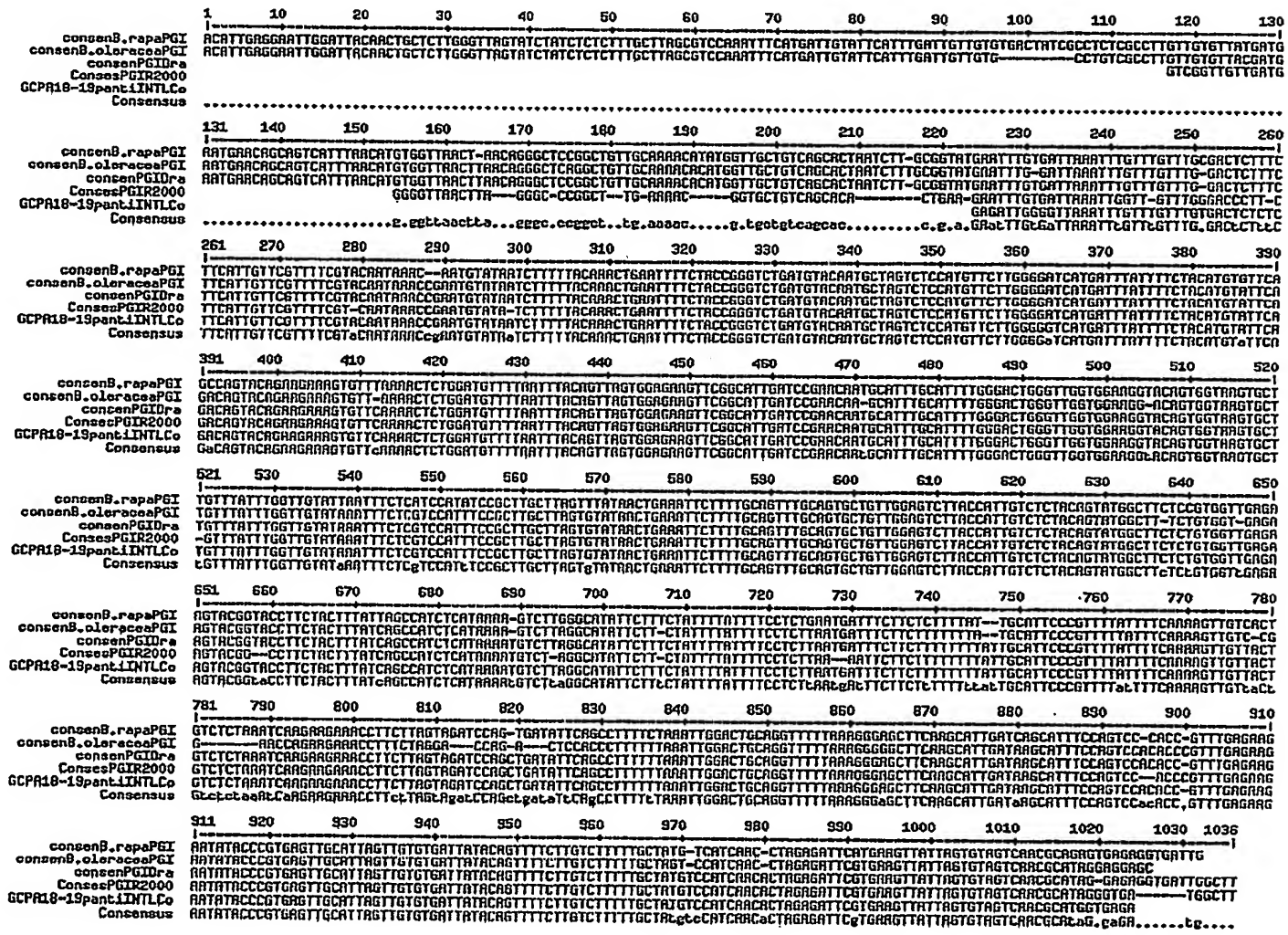
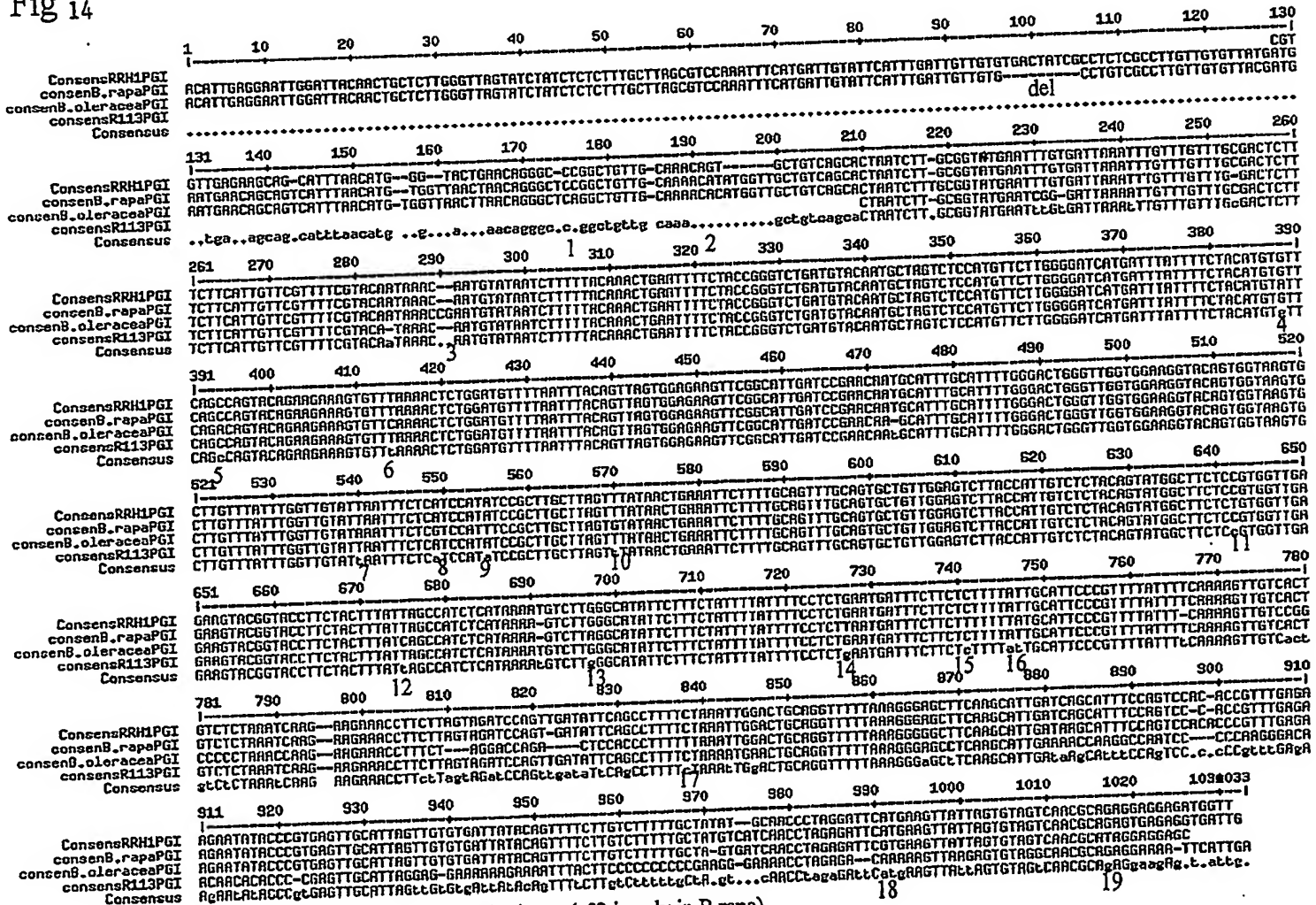

consenB.rapaPGI
consenB.oleraceaPGI
consenPGIDra
ConsecPGIR2000
GCPA18-19pantiINTLCo
Consensus

Fig 14



* one deletion and 19 substitutions, (n°2 is only in B.rapa)

Fig 15

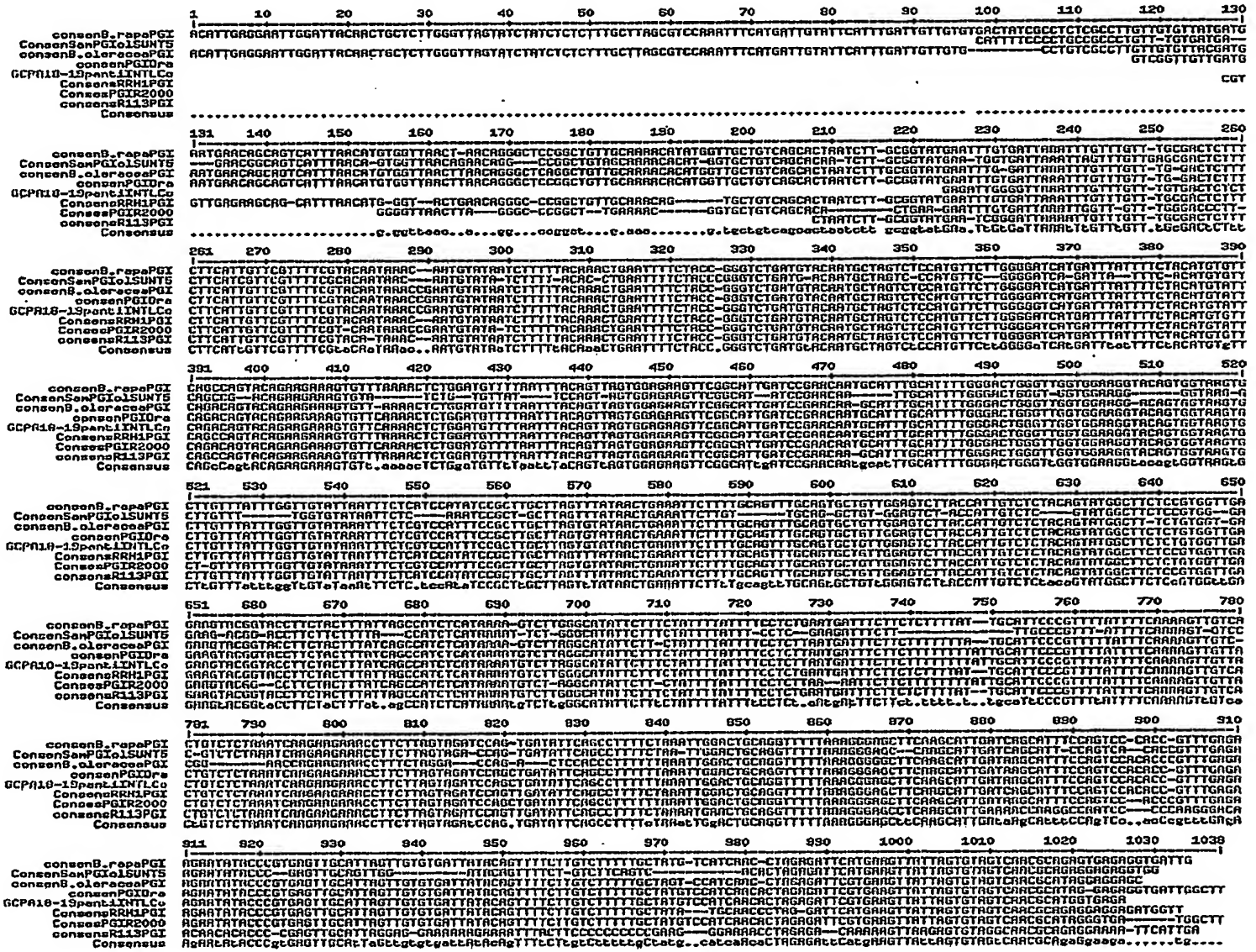
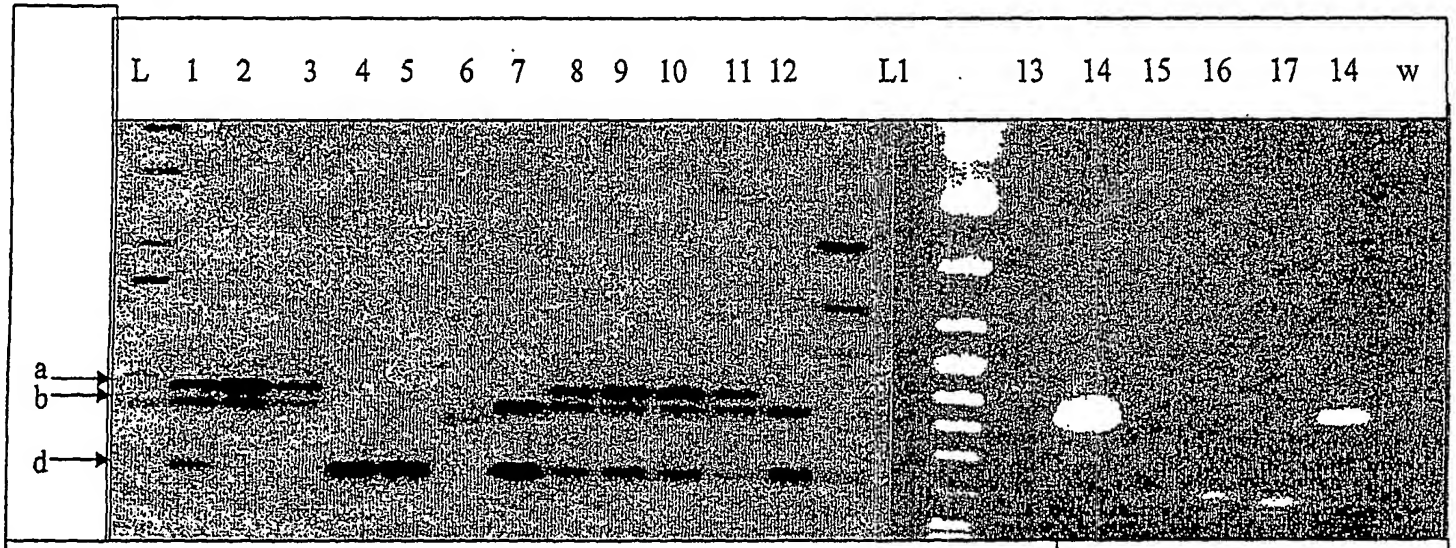
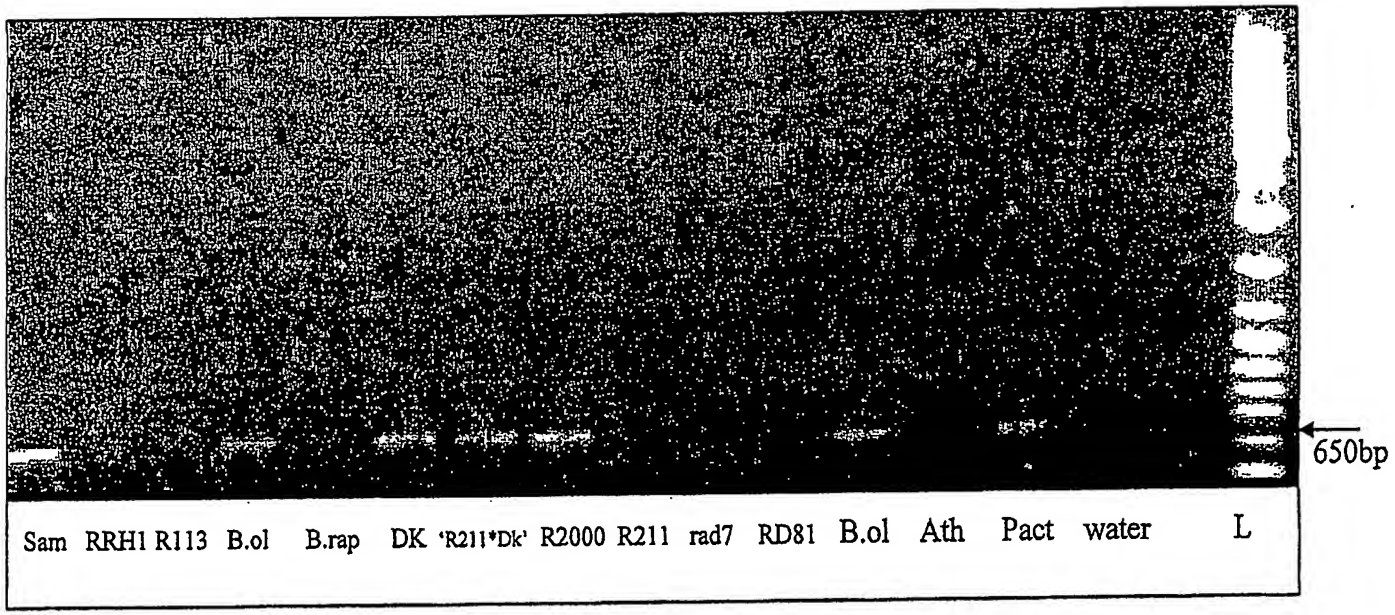

consenB.repaPGI
ConsenSanPGIolSUNT5
consenB.oleraceaPGI
consenPGIDra
GCPA18-19pantIINTLCo
ConsenaRRH1PGI
ConseaPGIR2000
consensR113PGI
Consensus

Fig 16



BolJon PCR bands (bp) n°		a	b	c	d	e
4,5	radish species:line 7				600	
17	radish RD81					590
14	B.rapa species		870			
13	B.oleracea species	950				
6	Arabidopsis thaliana (WS)			800		
2, 3	maintainer rapeseed: 2 Samourai; 3 Pactol	950	870			
7,12	homozygous restored rapeseed: 7 Samourai RRH1, 12 R211	0	870		600	
11	incomplete restored SRfi	950	870		600	
1,8	heterozygous restored rapeseed: R211.Dk	950	870		600	
9,10	homozygous restored R2000: 6 lineE38, 8,18,19 line R1.15	950	870		600	
L , L1	ladder1kb+, Ladder 100bp					

Fig 17



The CP418 band (about 650bp) specific to the B.oleracea genome.
It is present in B.ol, B.napus (Samourai, Drakkar, Pactol and the herterozygous R211*Dk)
It is absent from the restored rapeseed (RRH, R113 and R211)
It is present in the homozygous R2000.

Fig 18

Marker	size bp	N*WRadrad	RD81rad	line7	Sam s	RRH1	R113	R211	R2000	R211*Dk	Drakkar	Wesroon	B.oleracea	B.rapa	Arabid.th
PGIol	278	+	-	-	+	+	+	-	+	+	+	+	+	-	-
type B. ol	*278*							-	+	+	+	+	+		
type B.ra	*278*				+	+	+	-						+	
PGI UNT	950	+	-	-	+	+	+	-	+	+	+	+	+	-	-
type B. ol	*950*					-	-	-	+	+	+	+	+		
type B.ra	*950*				+	+	+	-						+	
PGI int															
radis	950	1000		+	-	+	+	-	-	-	-	-	-	-	-
Brassica	850	+		-	+	+	+	-	+	+	+	+	+	+	-
type B. ol	*850*							-	+	+	+	+	+		
type B.ra	*850*				+	+	+	-						+	
BolJon															
B. ole	950				+	-	-	-	+	+	+	+	+		
B.rap	870				+	+	+	+	+	+	+	+		+	
Ath	800														+
radis	600			+	-	+	+	+	+	+	-	-	-	-	
radis	590		+												

*Brassica*band characterised by the DNA sequence